Review

Clinicopathological, genomic and immunological factors in colorectal cancer prognosis

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Background: **Numerous factors affect the prognosis of colorectal cancer (CRC), many of which have long been identified, such as patient demographics and the multidisciplinary team. In more recent years, molecular and immunological biomarkers have been shown to have a significant influence on patient outcomes. Whilst some of these biomarkers still require ongoing validation, if proven to be worthwhile they may change our understanding and future management of CRC. The aim of this review was to identify the key prognosticators of CRC, including new molecular and immunological biomarkers, and outline how these might fit into the whole wider context for patients.**

Methods: **Relevant references were identified through keyword searches of PubMed and Embase Ovid SP databases.**

Results: **In recent years there have been numerous studies outlining molecular markers of prognosis in CRC. In particular, the Immunoscore® has been shown to hold strong prognostic value. Other molecular biomarkers are useful in guiding treatment decisions, such as mutation testing of genes in the epidermal growth factor receptor pathway. However, epidemiological studies continue to show that patient demographics are fundamental in predicting outcomes.**

Conclusion: **Current strategies for managing CRC are strongly dependent on clinicopathological staging, although molecular testing is increasingly being implemented into routine clinical practice. As immunological biomarkers are further validated, their testing may also become routine. To obtain clinically useful information from new biomarkers, it is important to implement them into a model that includes all underlying fundamental factors, as this will enable the best possible outcomes and deliver true precision medicine.**

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Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide[1], yet accurate prognostics evade us, often due to the heterogeneous nature of the disease. Current excitement about single fashionable factors, such as Oncotype DX® (Genomic Health, Redwood, California, USA), consensus molecular subtypes, the Immunoscore® (Integrative Cancer Immunology Laboratory, INSERM, Paris, France), tumour budding and stromal content, belies the complexity of the disease[2–5]. These factors may be insufficiently validated by limited studies of inadequate patient numbers, lack of robust comparison against standard prognosticators, or being tested only in trial populations. Most importantly, they rarely consider the individual characteristics of the patient, the tumour, the treating team or the expected site of recurrence (*Fig. 1*). Prognosis is improving with more access to screening and targeted oncology treatments that have reduced metastatic disease burdens. Delivery of precision cancer care requires sophisticated prognostic modelling that accounts for all these levels of complexity in differing socioeconomic populations and healthcare settings.

The anatomical extent of the disease, as assessed by clinicopathological staging, remains the most informing aspect of prognostic estimation. However, there are many patients whose outcomes do not match those typical for their tumour stage. Understanding the mechanisms behind these discrepancies will allow for a more precise, personalized approach, better informed treatment options, and

Fig. 1 Prognostic model for colorectal cancer, showing the many factors that influence outcome. PNI, perineural invasion; EMVI, extramural vascular invasion; MMR, mismatch repair status; MDT, multidisciplinary team; NLR, neutrophil/lymphocyte ratio; CMS, consensus molecular subtypes

ultimately improved outcomes. Over recent years, new evidence has impacted positively on the understanding of individual prognosis. This includes the importance of specific patient characteristics, the effectiveness of the multidisciplinary team, refinement of histopathological morphological factors, new molecular markers, and immunological indicators. Much of the immunological and molecular information provides promising new ways to classify and understand the prognosis of CRC, but the knowledge base of their relative importance remains incomplete. This review details key prognosticators of CRC, including new molecular and immunological biomarkers, and outlines how these might fit into the wider context for patients.

Methods

Searches of the PubMed and Embase Ovid SP databases were conducted for keywords 'colon' or 'rectum' or 'colorectal' and 'cancer' combined with 'prognosis' or 'outcomes', for papers published between July 2007 and July 2017. English-language references from these searches were then uploaded into a database, which was further interrogated for keywords in specific key areas such as 'biomarkers, immunology, molecular markers'. Additional relevant papers published before 2007 were identified through review articles.

Patient factors

It is a fundamental principle that prognosis and appropriate management in CRC is strongly related to individual patient characteristics. These include age, sex, co-morbidity and socioeconomic status. CRC is often a disease of the elderly, with the incidence increasing with age. In the UK, more than 44 per cent of new cases are diagnosed in those aged 75 years or over, and the peak incidence is seen in the 85–89 years age group[6]. It is thought that this is due to an accumulation of aberrant

genetic changes and a loss of the body's tumour defence systems with time. Co-morbidity and the patient's overall health should have a greater influence than absolute age, but there tends to be negative bias in the clinical decision regarding treatment (chemotherapy) regardless of overall state[7–9]. Elderly patients are poorly represented in clinical oncology trials and the benefit to elderly patients may be underappreciated[7,10,11].

It is also observed that women more frequently have right-sided cancers with microsatellite instability (MSI) and are less likely to have a rectal cancer than men. They have a survival advantage that may be due in part to oestrogen exposure, although the mechanisms are still unclear. It is known that women have a lower neutrophil/lymphocyte ratio than men, which may contribute to better long-term survival[12]. A mutation in the *p16* gene is found more often in right-sided tumours, and this mutation is nine times more frequent in all CRC in women than in men[13–15]. However, the mechanism behind this finding has yet to be fully explored. Women are more likely to present in an emergency situation with CRC, an independently poor prognostic factor. However, overall, women still have a better long-term survival, even when accounting for these and other differences in disease extent and treatment[16,17].

Levels of accompanying co-morbid disease represent an important influence on CRC prognosis. This is due not only to the increased risk of death from non-cancer causes but also to the influence on disease-specific mortality[9,18,19]. To account for the influence that co-morbidity may have on outcome, a number of classification systems are used in clinical practice. One of more commonly used methods in epidemiological studies is the Charlson co-morbidity index (CCI)[20]. This scoring system classifies co-morbidities according to the patient's risk of 1-year mortality. The CCI score has been used in numerous clinical trials and studies to enable multivariable analyses to account for co-morbidities influencing the effect of the studied intervention. Another commonly employed method to quantify the effect of co-morbid disease on a patient's frailty is to classify performance status (PS). The most commonly used PS systems are the WHO PS (also known as the Zubrod or Eastern Cooperative Oncology Group scale) and the Karnofsky score[21,22]. These scoring systems differ from methods such as the CCI in that they focus on the effect of co-morbidities on the ability to perform activities of daily living rather than the diagnosed condition itself. Scoring systems are blunt measures of quantifying co-morbidity. However, by using these systems, trials can provide more clinically useful information and prognosis can be assessed more accurately.

Another highly important patient characteristic is socioeconomic status, which is independently associated with prognosis[23–26]. Similar to co-morbid state, socioeconomic status is categorized by a number of different classification and scoring systems. In most epidemiological studies, socioeconomic groups are determined by the location of the patient's home and/or educational level. This means that they may not reflect the patient's true socioeconomic status, and this has to be considered in the interpretation of studies that use this method. Socioeconomic status can be viewed as a surrogate for prognosis rather than as a prognostic factor. For example, patients with a poorer socioeconomic status may have higher levels of co-morbidity, poorer diet, present with late disease, be more likely to present as an emergency, have high levels of smoking, and so on. These factors may be the influence on poor prognosis rather than socioeconomic status itself[27].

Surgery and the multidisciplinary team

The multidisciplinary team delivering care and treatment to the patient has a significant influence on prognosis, regardless of the molecular make-up of the tumour and patient factors. Surgery is often the main curative component of treatment, especially for earlier stage tumours. The plane of surgery has been demonstrated as an important prognostic factor for recurrence following both rectal and colonic cancer resections[28,29]. In rectal cancer, it has been demonstrated[30] that the presence of tumour cells at or within 1 mm of the surgical circumferential resection margin is an independent poor prognostic factor. Involvement of the circumferential resection margin is dependent on the extent of the disease, the effectiveness of radiological techniques in its prediction, and the quality of surgery. Total mesorectal excision involves full excision of the mesorectum in an intact fascia-lined package, thereby achieving the best plane of surgery, and the lowest incidence of involved margins and local recurrence[31,32]. High-quality, standardized surgery for low rectal cancer and colonic cancer can have an effect on outcomes[33–36].

From a radiological perspective, staging accuracy has a significant impact on subsequent surgical outcome. Improved imaging technologies have allowed for more accurate staging and therefore more accuracy in predicting prognosis. In addition, optimal planning of surgical approaches relies on robust imaging, which leads to oncologically superior outcomes whilst limiting morbidity (where possible). MRI, combined with CT, is a robust method for accurate prediction of surgical margin involvement[37,38].

For rectal cancer, radiotherapy is now a well established treatment modality in addition to surgery. It reduces the risk of local recurrence, although short-course radiotherapy may not influence overall survival. There are therefore differences in its application across the world. In the USA and Europe, the most common approach is preoperative short-course radiotherapy; however, in Japan this is not standard practice[39,40]. In England, wide variation has been demonstrated[41] in the use of radiotherapy, with men and patients having an abdominoperineal excision receiving it more often, and elderly patients and those with co-morbidities less so. This will have an impact on potential side-effects and local recurrence rates, and therefore may influence prognosis.

There is a strong association between the total number of lymph nodes examined after resection and overall survival. A large clinical trial[42] showed that for node-negative patients, median 5-year survival was significantly improved when over 20 lymph nodes were identified. This is dependent on many factors, including patient immunology, tumour biology, anatomical location in the bowel, age, sex, preoperative treatment, and the quality of the surgery and the pathological assessment.

Histopathologists may face challenges when assessing the morphology of CRC specimens; the histological features are often subjective and opinion may vary between assessors. An example of this is when establishing the tumour grade. Grading tumours involves classifying the degree of cellular differentiation. The WHO grading system[43] consists of four subcategories based on the percentage of glandular differentiation, whereby grade 1 denotes well differentiated tumours, grade 2 is moderate differentiation, grade 3 is poor differentiation, and grade 4 is undifferentiated. It is well established[44,45] that tumour grade is significantly associated with survival. However, for day-to-day use a two-grade system of poor or other is more helpful as an aid to decision-making, as this improves interobserver agreement. Algorithms that automatically and consistently grade tumours need development, potentially by machine learning to improve the benefit of this important feature.

Another morphological feature that can be difficult to assess is the depth of tumour invasion, which has long been established as a prognostic marker. For a depth of submucosal invasion greater than 1 mm in pT1 cancers, there is a significantly higher risk of lymph node metastasis[46,47]. As tumours grow and invade through subsequent layers of the bowel, there may eventually be peritoneal involvement. This has independent adverse prognostic significance and it is therefore important to classify these tumours accurately. However, on histopathological examination it can be difficult to identify peritoneal involvement, which is therefore subjective with the potential to be missed. A study[48] that involved cytological examination of serosal scrapings identified that 26 per cent of tumours classified as pT3 contained malignant cells. Newer methods of assessing peritoneal disease might be valuable.

Tumour pathology

The tumour itself provides a large amount of information that holds important value for prognosis. This includes studying macroscopic and microscopic histopathological features, as well as the molecular signature. The stage at which a tumour is diagnosed and the grade of differentiation provide fundamental information regarding likely prognosis to guide management. The AJCC and the UICC TNM staging system informs clinicians about the extent of tumour growth and spread into lymph nodes and/or distant metastatic deposits. It is continually reviewed and updated, as the role and impact that diagnostic features have on outcomes are further understood. Important histopathological features that are independent prognostic markers include depth of invasion in the bowel wall, peritoneal involvement, proportion of tumour cells/stroma in a given area, perineural and lymphovascular invasion, extramural venous invasion, tumour deposits and tumour budding[49]. One notable criticism of the TNM system is that it is frequently based on retrospective data from limited patient populations and from mainly within the USA, which may or may not be representative of healthcare systems around the world.

CRC consists of malignant epithelial cells mixed with benign stroma comprising fibroblasts, lymphatic and vascular structures, and inflammatory cells. Studies[50,51] have shown that the proportion of tumour cells within a tumour area is an independent prognostic marker, and increases with stage. This is thought to be due to the cross-talk between stroma and carcinoma cells producing a greater level and number of growth factors, possible protection of tumour cells from immune attack, and the inverse association with deficient mismatch repair[51,52].

The presence of vascular or perineural invasion has long been recognized as a prognostic factor[45,53]. Given its clinical impact, perineural invasion was added to the seventh edition of the AJCC TNM staging manual[49]. Another factor that has prognostic value but that may be difficult to determine on pathological assessment is direct invasion of the tumour into blood vessels. This is determined as intramural invasion when it occurs in the submucosa and/or

muscular layer, and as extramural venous invasion when it invades beyond the muscularis propria[54].

An important component of TNM staging is the detection of tumour cells within lymph nodes. Occult tumour cells within lymph nodes have previously been categorized as micrometastasis when 0·2–2 mm in size or as isolated tumour cells when less than 0·2 mm. Meta-analyses[55,56] have shown that, for stage II and III tumours, micrometastases are associated with worse prognosis but isolated tumour cells are not. This is reflected in TNM staging, whereby micrometastases are counted as an involved node, whereas isolated tumour cells are included in the pN0 category[49].

Tumour deposits are an important prognostic factor. They refer to a focus of tumour in the pericolic/perirectal fat within the lymph drainage area of the primary tumour but without identifiable lymph node, neural or vascular structure. Their presence as an adverse prognostic marker has been recognized since the fifth version of the AJCC TNM staging manual, but their definition has been refined over time. Although still suboptimal with the potential for subjectivity, there is no doubt their description adds value to TNM[57,58]. A recent meta-analysis[59] suggested that the presence of both tumour deposits and lymph node metastases is additive in indicating a poor prognosis, possibly suggesting the presence of more than one metastatic pathway.

A histopathological feature that is not currently part of the TNM staging system is tumour budding. This refers to individual or small discrete clusters of tumour cells (fewer than 5) present at the invasive edge of the tumour. Its underlying mechanism is not fully understood, but is thought to represent an epithelial–mesenchymal transition whereby cell adhesion is lost, there is resistance to apoptosis, and cells gain an invasive phenotype[60]. Tumour budding is strongly associated with a number of poor prognostic histopathological factors, including higher tumour grade, lymphovascular invasion, lymph node metastasis and distant metastasis. It has been shown to be a strongly independent prognostic factor in numerous studies[5,60–62]. Despite extensive evidence for its usefulness as a marker of prognosis, until recently there has been a lack of consensus in the practical assessment of tumour budding, leading to subjectivity in the interpretation. Factors to be considered include the best topographical area for assessment, the microscopic field number and size that should be used, and whether staining should be with haematoxylin and eosin or by immunohistochemistry. However, work is ongoing to address this, including standards set by the International Tumor Budding Working Group so that this marker can be incorporated into staging in the future[62].

Molecular markers

There are numerous prognostic molecular markers for CRC at all levels, including DNA, RNA and protein (*Table 1*)[63]. DNA can be affected in multiple ways in cancer pathways; this includes small-scale changes such as mutations, deletions and insertions, and larger changes such as methylation, MSI and chromosomal rearrangements.

The 'classical' model of CRC tumour development is from normal mucosa, through to adenoma and then carcinoma, and it is thought that the majority of cancers develop in this way. At the molecular level, the model consists of early loss of regulation of the Wnt signalling pathway, followed by accumulation of activating mutations in oncogenes such as *KRAS*, *PIK3CA* and *BRAF*. Malignant transformation is then thought to occur through mutations in genes such as *TP53* and *SMAD4*, and via chromosomal instability. Although, at present, none of these individual molecular events is used as a clinical prognostic marker, the overall chromosomal instability phenotype is associated with worse survival in comparison with that in patients with MSI tumours, as also found in studies looking at DNA aneuploidy, a more crude assessment of DNA content[64–66].

Within CRC a number of biological classifications have been proposed: those cancers with MSI, CpG island methylator phenotype (CIMP) or chromosomal instability, and more recently based on RNA profiles into consensus molecular subtypes I–IV[3,67,68]. Although these biological subtypes also correlate with prognosis, there is not yet sufficient detailed information to identify their true place in clinical practice compared with other important parameters. There is also considerable overlap of these with other prognostic markers. Large-scale international collaborations on populations are needed to truly define the role of what can be commercially expensive tests.

Although they may have limited use as prognostic markers, mutations are valuable as predictive markers for guiding treatment. In metastatic CRC there are drugs that target the epidermal growth factor receptor (EGFR) such as panitumumab and cetuximab, but for patients with tumours that have activating mutations of the downstream RAS proteins, targeting EGFR does not improve outcomes. Studies[69–71] have shown consistently that in tumours with *KRAS* mutations, there are poorer progression-free and overall survival rates when treatment includes anti-EGFR therapy compared with those in patients with tumours that are wild-type for *KRAS*. More controversy exists over mutations in *BRAF* and *PIK3CA*, EGFR amplification, amphiregulin and epiregulin RNA levels, and markers such as PTEN (phosphatase and tensin homologue) protein expression[70,72,73].

Table 1 Summary of recommended clinical molecular biomarkers in colorectal cancer[63]

Biomarker	Mechanism	Use
KRAS	EGFR signalling pathway	Gene mutation status. For patients to be considered for anti-EGFR monoclonal antibodies
NRAS	EGFR signalling pathway	Gene mutation status. For patients to be considered for anti-EGFR monoclonal antibodies
BRAF	EGFR signalling pathway	Gene mutation status. For prognostic stratification and for dMMR tumours with loss of *MLH1* to evaluate risk of Lynch syndrome
Mismatch repair status	DNA mismatch repair	Immunohistochemistry or MSI testing. For prognostic stratification and screening of Lynch syndrome

EGFR, epidermal growth factor receptor; dMMR, DNA mismatch repair; MSI, microsatellite instability.

Although it is considered that the majority of tumours develop via chromosomal instability, approximately 12–15 per cent are deficient in DNA mismatch repair (dMMR) and therefore have high levels of MSI. This loss of MMR can occur through germline mutations, known as Lynch syndrome, or sporadic epigenetic silencing and CIMP of the MMR genes. Lynch syndrome is associated with a high risk of developing metachronous tumours, and a more extensive surgical approach such as extended colectomy is therefore often recommended[74,75].

Approximately half of sporadic CRCs with dMMR also carry a mutation in the *BRAF* gene. There are also phenotypic associations seen with dMMR tumours such as a proximal location within the colon, female sex, poor differentiation, mucinous histological phenotype and higher levels of lymphocytic infiltration. Tumours with dMMR are associated with better stage-adjusted survival compared with that in proficient MMR tumours[76–78]. They also have a decreased risk of metastasizing and are therefore associated with earlier stage[79]. The increased antigen-driven immune response caused by dMMR is thought to be part of the reason behind this. It has also been suggested[80] that in dMMR the β2-microglobulin gene is often mutated in its microsatellite coding regions. This results in an inability to present antigens at the cell surface, which, in turn, stimulates natural killer cell-mediated tumour cell death[80].

MMR status is not only a useful prognostic tool, but also may have a role in predicating response to therapy. For stage II dMMR tumours, some clinical trials have shown that 5-fluorouracil (5FU)-based adjuvant chemotherapy may not provide clinical benefit; however, this has not been found in all clinical trials and its clinical significance is therefore unclear[81]. In stage III disease, treatment with adjuvant FOLFOX (folinic acid, 5FU and oxaliplatin) is recommended regardless of MMR status. As well as traditional chemotherapy, more recently there have been advances in the treatment of dMMR tumours with immunotherapy. These tumours often have increased expression of immune checkpoints including programmed death (PD) 1. A phase 2 clinical trial of the monoclonal antibody to PD-1, pembrolizumab, has shown high rates of response in MSI tumours, and the 20-week progression-free survival rate was up to 78 per cent, compared with no response in proficient MMR tumours[82]. There are ongoing trials to explore further other immune checkpoint targets for dMMR tumours.

Assessment of MMR status has a number of clinical implications including understanding prognosis, an increased incidence of metachronous cancers, differential response to treatment (especially immunotherapy), and the identification of patients at higher risk of Lynch syndrome. It is recommended by the National Institute for Health and Care Excellence[83] and in the eighth version of the AJCC TNM staging manual[49], but is not currently widely implemented in routine testing in the UK. This needs to change rapidly.

RNA includes both coding and non-coding RNAs (ncRNAs) such as microRNAs and long non-coding RNAs amongst others. MicroRNAs regulate gene expression, and their dysregulation is associated with a number of malignancies[84]. There has been extensive work to understand and classify the functions of ncRNAs within CRC. Many ncRNAs have been shown to be independent prognostic factors and are associated with later stages of CRC, and therefore a worse prognosis[85,86]. However, the true prognostic potential of ncRNAs has yet to be fully explored and their relative value translated through to clinical practice.

Immunological factors

The immunological status of a patient with CRC can have a significant impact on their prognosis. There may be systemic inflammation, and host response factors also play a key role locally within the tumour. On a systemic level, a marker of inflammation that is increasingly used is the neutrophil/lymphocyte ratio (NLR), calculated by dividing the neutrophil count by the lymphocyte count. An increased NLR is seen with lymphocytopenia and neutrophilia. Lymphocytopenia indicates impaired cell-mediated immunity, whereas neutrophilia is seen as an acute inflammatory response. A raised NLR, and

therefore higher levels of systemic inflammation, have been associated with worse prognosis in numerous studies, and a recent meta-analysis[87] showed that an increased NLR was associated with significantly shorter overall and progression-free survival rates. NLR is an easily measured, cost-effective marker that has a significant association with outcome in a number of solid tumours, and it may hold greater clinical impact in the future. The additional value of other immune markers needs to be understood in the context of this very cheap routine test.

The immune system also plays an important role locally within CRC. Numerous immune cell types are found in tumours; in particular, higher levels of lymphocytic infiltrate are associated with improved outcomes. The lymphocytic infiltrate can be profiled using immunohistochemistry for T cell markers, such as cluster of differentiation (CD) 3, CD4, CD8 and FOXP3. Studies have shown that the presence of CD3 cytotoxic T cells and CD45RO memory T cells is a strong marker of prognosis. For patients with low densities of CD3 and CD45RO cells, both in the tumour core and in the invasive margin, it has been demonstrated[88] that, regardless of stage, their overall survival is similar to that in patients with stage IV tumours. Standardized scoring of the immune infiltrate in this way, denoted as the Immunoscore® by Fridman and colleagues[89], has been suggested as a clinically useful prognostic marker owing to its strong association with outcome in numerous studies. However, its implementation requires ongoing independent validation with retrospective case series and further work to identify how it relates to the MSI subset of tumours. As this method continues to be standardized it has the potential to be included in TNM staging[4]. Interestingly, assessment of the total number of lymphocytes present may also be an effective method of predicting prognosis, as shown for other cancer types, although further work is needed to explore its role in CRC[90].

Conclusion

Current strategies for managing CRC are strongly dependent on clinopathological staging. Staging systems are continually reviewed and updated to include new validated markers of prognosis. A large number of new markers and further markers are emerging – not only histopathological features, but also molecular and immunological data. Some of these hold a strong association with prognosis, but it is important not to forget the greater context for the individual patient. A patient's tumour may have excellent biological markers, but if the person is elderly, has many co-morbidities, is socioeconomically deprived or is managed outside a high-quality multidisciplinary team by a non-specialist surgeon then the isolated biological profile may not hold as much relevance for overall prognosis. In the future, it is vital to understand how we can use this new biological information in combination with current established markers, patient demographics and data on the effectiveness of treatment. This can be undertaken only by the creation of new international networks that can generate high-resolution, high-quality, anonymized, large public data sets from different healthcare systems. These can then be used to model CRC more accurately and deliver truly precision medicine for the individual patient.

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